Available online at www.sciencedirect.com

ScienceDirect

Biochimica et Biophysica Acta 1773 (2007) 1447–1454

Biochimica et Biophysica Acta BBA

www.elsevier.com/locate/bbamcr

Alien inhibits E2F1 gene expression and cell proliferation

Stephan P. Tenbaum [a,1,2], Maria Papaioannou [a,b,1], Christina A. Reeb [a,b], Frauke Goeman [a,3], Niko Escher [c], Robert Kob [c], Ferdinand von Eggeling [c], Christian Melle [c], Aria Baniahmad [a,b,*]

[a] *Molecular Genetics, Institute of Human Genetics and Anthropology, Friedrich-Schiller-University, 07740 Jena, Germany*
[b] *Department of Biochemistry, University Kuopio, Finland*
[c] *Core Unit Chip Application (CUCA), Institute of Human Genetics and Anthropology, Friedrich-Schiller-University, 07740 Jena, Germany*

Received 23 January 2007; received in revised form 25 April 2007; accepted 28 April 2007
Available online 10 May 2007

Abstract

Recently, using a proteomic approach we have identified the corepressor Alien as a novel interacting factor of the cell cycle regulator E2F1. Unclear was whether this interaction influences cell proliferation and endogenous E2F1 target gene expression. Here, we show by chromatin immunoprecipitation (ChIP) that Alien is recruited *in vivo* to the E2F binding sites present in the E2F1 gene promoter, inhibits the transactivation of E2F1 and represses endogenous E2F1 gene expression. Interestingly, using synchronized cells to assess the expression of Alien profile during cell cycle the levels of endogenous Alien are increased during G1, G1/S and G2 phase. Furthermore, stable transfection of Alien leads to reduction of cell proliferation. Thus, the data suggest that Alien acts as a corepressor for E2F1 and is involved in cell cycle regulation.

Keywords: Corepressor; Gene repression; Transcription; E2F1; Cell cycle

1. Introduction

The transcription factor E2F1 plays a crucial role in the regulation of cell-cycle progression at the G1-S transition [1–3]. Furthermore, E2F1 is associated with DNA repair and apoptosis [4–8]. In line with that, deregulation of the transcription factor E2F1 is a common event in most human cancers. E2F1 is a transcriptional activator and a member of the E2F transcription factor family that regulates cell cycle progression ([7,9–11]). As the other members, E2F1 regulates the expression of a number of cell cycle regulatory factors. Interestingly, E2F1 itself induces the expression of its gene by E2F binding sites located in the promoter region [12]. E2F1 forms protein complexes with other cellular proteins that are known or inferred to regulate cell cycle. E2F1 mediates its transcriptional activation through binding to coactivators ([13,14]). Moreover, it has been shown that E2F-1 transcriptional activity, DNA binding, and protein stability are regulated by protein acetylation [15].

Other interacting factors such as the retinoblastoma protein (pRB) and the pocket domain protein p107 are known to bind directly to and repress E2F1 transcriptional activation [16–19]. One mechanism of pRB-mediated repression of E2F1 is through association with histone deacetylase activity (HDAC) and histone methyltransferase activity [20,21].

Endogenous E2F1 message abundance is regulated in a cell cycle dependent manner. E2F1 seems not be abundantly present in resting cells [22]. Deregulation of E2F1 is a common event in many human cancers [4] and overexpression of E2F1 can advance cells from quiescence into S-phase [22,23,7] indicating that lack of E2F1 downregulation and lack of E2F1-mediated

Abbreviations: ChIP, chromatin immunoprecipitation; CoIP, co-immunoprecipitation; IB, immunoblot; IP, immunoprecipitation; MS, Mass spectrometry; pRB, retinoblastoma protein; T3, thyroid hormone; TR, thyroid hormone receptor; tTA, Tet activator; VDR, vitamin D3 receptor

* Corresponding author. Institute of Human Genetics and Anthropology, Medical Faculty, Friedrich-Schiller-University, 07740 Jena, Germany. Tel.: +49 3641 935524; fax: +49 3641 934706.
E-mail address: aban@mti.uni-jena.de (A. Baniahmad).
[1] These authors contributed equally to this work.
[2] Present address: Molecular Oncology, CNIO, Madrid, Spain.
[3] Present address: Laboratorio di Oncogenesi Molecolare, Instituto Regina Elena, Rome, Italy.

0167-4889/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2007.04.017

transactivation is one important factor for cell proliferation. Analysis of the cell cycle dependent expression profile of E2F1 after growth stimulation suggests it is expressed throughout the cell cycle with highest expression detected in S-phase [24,25].

Interestingly, a complex containing E2F1 and the corepressor Alien was very recently detected [19]. This interaction was confirmed by *in vitro* binding assays suggesting a direct binding of E2F1 and Alien. Alien was previously characterized as a corepressor for specific members of the nuclear hormone receptor superfamily including the thyroid hormone receptor (TR), vitamin D3 receptor (VDR) and DAX1 [26–28]. Alien interacts in a hormone-sensitive manner with TR and VDR and enhances transcriptional repression. One mechanism of Alien-mediated repression was shown to be mediated by HDAC activity [26]. Here, we followed the notion whether Alien might act as a corepressor for E2F1 transcriptional function.

2. Experimental procedures

2.1. Cell culture and DNA transfection

Cell lines used here were cultured in DMEM supplemented with 10% fetal bovine serum whereas the LNCaP cell line was cultivated in RPMI and supplemented with 10% fetal bovine serum. Transient transfection experiments were performed according to Moehren et al. [29]. The experiments have been repeated at least three times. Tetracycline regulation was performed using the NIH3T3-S2-6 cells [30] containing the Tet-activator (tTA). Cells were grown in the presence of 0.5 μg/ml tetracycline and 0.5 mM histidinol [30] and infected retrovirally with pHRS vectors using cells expressing the ecotropic receptor [31]. Stable selected cell clones were selected 2 days post transduction with 2 μg/ml puromycin. Cell numbers were counted in the absence or presence of tetracycline (0.5 mg/ml). Stable transfection experiments with LNCaP cells [32] were performed using DOTAP transfection reagent according to the manufacturer's protocol (Carl Roth GmbH, Karlsruhe, Germany). Serum-free RPMI medium (Invitrogen) was used to transfect cells with pETE-Hyg control plasmid [32] or pETE-Hyg-Alien expression vector (5 μg each for a 10-cm dish). 24 h after transfection, the medium was changed and replaced with RPMI containing 5% serum. After a further cultivation of 48 h, selection of transfected LNCaP cells was accomplished by addition of 200 μg/ml hygromycin into the culture medium. Cells were cultured for approximately 2 weeks, changing the medium and hygromycin every 2–3 days until untransfected control cells were completely killed by the selection.

2.2. ChIP

After 3 days of cultivation of LNCaP cells with RPMI supplemented with 2% charcoal treated FCS, the proteins were cross-linked to DNA by adding formaldehyde directly to the medium resulting in a final concentration of 1% at 37 °C for 10 min. Cross-linking within the cells was stopped by adding glycine at a final concentration of 0.125 M and incubating at room temperature (RT) for 5 min on a rocking platform. Cells then were rinsed twice with ice-cold PBS, collected into ice cold PBS supplemented with a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). After centrifugation the cell pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA 50 mM Tris–HCl pH 8.0 and protease inhibitors). The lysates were sonicated on ice 10 times, 10 sec at 10% of maximum of power (Branson W-250/W), to yield DNA fragments of 500 bp in length. After centrifugation supernatants were collected and diluted in ChIP dilution buffer (0.01% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–HCl pH 8.0) followed by preclearing with 30 μl of salmon sperm DNA/protein A agarose 50% slurry (Upstate Biotechnology, Lake Placid, NY) for 1 h at 4 °C with agitation. Immunoprecipitation was performed overnight at 4 °C with the rabbit anti-androgen receptor antibody (Upstate Biotechnology) or with Alien specific antibody (PEP AK-2 [26]. The immunocomplexes were collected with 30 μl of salmon sperm DNA/protein A agarose 50% slurry for another 2 h with rotation at 4 °C. Agarose beads were pelleted by centrifugation and washed sequentially for 10 min each with 1 ml of the following buffers: light salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8, and 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8, and 500 mM NaCl), and LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, and 10 mM Tris–HCl, pH 8). Finally, the beads were washed two times with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8). The immunocomplexes were eluted twice from the beads by adding freshly prepared elution buffer (1% SDS, 0.1 M $NaHCO_3$). Eluates were pooled and the cross-linking was reversed by adding NaCl to final concentration of 200 mM and heated at 65 °C overnight. The remaining proteins and RNA were digested by adding proteinase K (final concentration 40 μg/ml) and RNase A (20 μg/ml) respectively and incubating at 55 °C for 3 h. The DNA fragments were purified with a DNA purification kit (QIAquick PCR Purification Kit, Qiagen, Hilden, Germany). For PCR, 5 μl out of 50 μl DNA was used in 34 cycles of amplification with addition of betain (final concentration 1 M). The primer sequences and the positions from transcription start sites are as follows:

E2F elements forward (−1429 bp): $^{5'}$ AGGAACCGCCGCCGTTGTTCCCGT $^{3'}$
E2F elements reverse (−1247 bp): $^{5'}$ GCTGCCTGCAAAGTCCCGGCCACT $^{3'}$
E2F1-upstream forward (−206 bp): $^{5'}$ CAGAACCGTGGTCTCCTTGTCACAGTC $^{3'}$
E2F1-upstream reverse (−105 bp): $^{5'}$ TCACTTCCTCTATTGCCCACTGCTGCC $^{3'}$

3. Quantitative real-time PCR (qRT-PCR)

2×10^6 LNCaP, as control, or LNCaP-Alien cells were seeded out per 10 cm dish in RPMI containing 10% charcoal-stripped FCS. 72 h later medium was replaced with fresh RPMI (10% charcoal-stripped FCS). RNA was isolated 24 h after hormone induction using peqGOLD TriFast (Peqlab) according to the manufacturer's protocol. 0.88 μg of RNA were used per sample in an one-step qRT-PCR reaction using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen) with these primer sequences:

E2F1-forward: $^{5'}$CAAGAAGTCCAAGAACCACATCC$^{3'}$
E2F1-reverse: $^{5'}$AGATATTCATCAGGTGGTCCAGC$^{3'}$
beta-actin-forward: $^{5'}$ACAGAGCCTCGCCTTTGCCGA$^{3'}$
beta-actin-reverse: $^{5'}$CACGATGGAGGGGAAGACG$^{3'}$

Data analysis was done by normalizing to beta-actin mRNA levels as described [33].

3.1. Synchronization of eukaryotic cells

To stop HeLa cells in different cell cycle stages, the cells were submitted to different treatments as described in Krek et al. [34]. For these assays, two equal 80% confluent 10-cm dishes were used for each cell cycle phase. One dish of each duplicate lysed for protein extracts for Western blot analysis, and the other one was ethanol-fixed for FAC-Scan analysis as described below. Mitosis: To stop cells in mitosis they were treated 16–18 h with 50 ng/ml nocodazol (Sigma). Mitotic cells with round morphology were detached by *mitotic shake-off*, harvested by centrifugation (10 min 1000 rpm, RT) from the medium. G1-phase: HeLa cells were synchronized in G1-phase by mimosine treatment (0.5 mM, for 24 h, Sigma). G1/S-phase: Treatment of the HeLa cells with hydroxy urea (Sigma;

2 mM, over night) accumulates cells at the G1/S-border of the cell cycle. S-phase/G2 phase: To stop HeLa cells in S-phase, the cells were submitted to thymidine block (Sigma; 2.5 mM over night). The cells were then washed twice with PBS to remove the thymidine and to release the cell cycle block and normal medium was applied. After growth of the cells for 2 h the S-phase was reached, after 8 h release the cells were in G2-phase.

3.2. Fixation and FAC-Scan analysis

To assay cell cycle stage of HeLa cells, the cells were harvested and ethanol-fixed. Thereafter, the medium was recollected and centrifuged (5 min, 2500 rpm, RT) to obtain detached apoptotic cells. The remaining cells were harvested using trypsin and centrifuged as mentioned above. The two cell pellets were pooled and the resulting pellet was washed twice in PBS. To fix the cells, 1 ml of ice-cold 70% ethanol (−20 °C) was added slowly, the cells were resuspended carefully and the probes were incubated for 15–30 min on ice. After that, the cells were spun down (pulse) and washed again with PBS, resuspended in 200 μl PBS and treated for 30 min at 37 °C with 1 μl RNase-A (10 μg/μl). Then, DNA was stained with 50 μg propidium iodide (PI, Sigma; 10 μg/μl) and cell cycle profile was acquired in a FACS Aria Cytometer (BD Bioscience).

3.3. Co-immunoprecipitation

The Co-IP assays were carried out as described [35]. Briefly, a specific antibody or, as negative control, normal rabbit IgG were bound on protein A-agarose beads. Crude extract (250 μl) from U-2OS cells was incubated with the antibody loaded beads for 1 h at 4 °C. Then the resins were washed three times with Co-IP buffer containing 20 mM HEPES/KOH pH 8.0, 50 mM KCl, 0.1 mM EDTA and 0.05% CHAPS. Bound proteins were subjected to 10% SDS-PAGE and detected by immunoblotting. (For details concerning used antibodies see figure legends).

3.4. Transient transfection

C33A cells were cultured and transfected in DMEM supplemented with 10% FCS. Cells were seeded 48 h prior to transfection into 6 well plates at a density of 240 000 cells per well. Medium was changed 9 h prior to transfection. DNA was introduced according to the CaPO4 transfection method described [29]. 0.5 μg E2F1-promoter-luc, E2F1mut-promoter-luc reporter-plasmids [12], or pUAS4xTATA-Luc [36], 0.7 μg indicated expression-vector and, 0.3 μg pCMV-LacZ per well were used and total amount of DNA was adjusted to 5.4 μg with calf thymus DNA. The expression vector for Gal-E2F1, expressing the E2F1 residues 284–437, was kindly provided by Dr. Bentley [37]. 24 h past transfection cells were washed 3 times with 2 ml PBS and medium was changed. After further 48 h of culturing cells were harvested and lysed to measure luciferase as well as beta-galactosidase activity for normalization and transfection efficiency control.

4. Results

4.1. Alien is recruited to the E2F1 promoter in vivo

The E2F1 gene expression is regulated by binding of E2F transcription factors to the E2F1 promoter [12]. Previously we have described the in vivo interaction of E2F1 with Alien in a proteomic approach using the combined techniques of immunoprecipitation and mass spectrometry (MS), which is confirmed in vitro and by co-immunoprecipitation ([19] and Fig. 1A). Immunoprecipitation with either an E2F1 or reciprocally with an Alien antibody [26] and subsequent immunoblot with E2F1 or Alien antibody, respectively, detected E2F1 (Fig. 1A, upper panel) or Alien (Fig. 1A, lower panel) as co-immunoprecipitate. Similar results were obtained using an E2F3 specific antibody suggesting that Alien is also complexed with E2F3 (data not shown and [19]). These data suggest an *in vivo* interaction of E2F1 with the corepressor Alien.

Unclear was whether Alien is recruited *in vivo* to chromatinized DNA. For that purpose we employed the ChIP-technique. Immunoprecipitation of Alien coprecipitated the genomic sequence of the E2F binding sites of the E2F1 promoter, which was detected by PCR (Fig. 1B upper panel). Unspecific antibodies, as negative controls, did not show a similar PCR band suggesting that Alien is recruited to the E2F1 promoter *in vivo*. Another negative control involved the analysis of upstream sequences of the E2F1 gene. Compared to the input, there is only a weak band observed using the anti-Alien antibody (Fig. 1B lower panel). The obtained data suggest that Alien is only weakly recruited to the upstream chromatin sites indicating a significant recruitment of Alien to the proximal E2F1 promoter part, which contains E2F binding sites.

4.2. Alien inhibits E2F-1 gene expression in vivo

To detect in a first step whether Alien influences the transcriptional activity of E2F1, E2F-responsive reporter assays were performed. For that purpose, the wildtype E2F1 promoter-luciferase reporter containing the E2F-binding sites from the E2F1 promoter together with expression plasmids coding for Alien or, as a control, the empty vector were transfected in C33A cells lacking the expression of functional retinoblastoma protein (Fig. 2A). As a further control, a reporter with the mutated E2F-binding sites within the same promoter context was employed [12]. Expression of Alien inhibited the reporter activity. As expected, using as control the reporter mutated at the E2F binding sites, which exhibited weaker transcriptional activity (data not shown), the co-expression of Alien did not repress as strongly as using the wildtype E2F binding sites (Fig. 2A). This suggests that the transcriptional activity of the E2F1 promoter is inhibited by Alien through the E2F binding sites. Since different E2F family members can bind to E2F sites it was unclear whether the transactivation of E2F1 itself can be repressed by Alien.

Thus, in a second step to verify whether Alien inhibits the transactivation function of E2F1, a Gal-E2F1 fusion was used together with the UAS-reporter (pUAS4x-TATA-Luc) and Alien expression vector was cotransfected with increasing

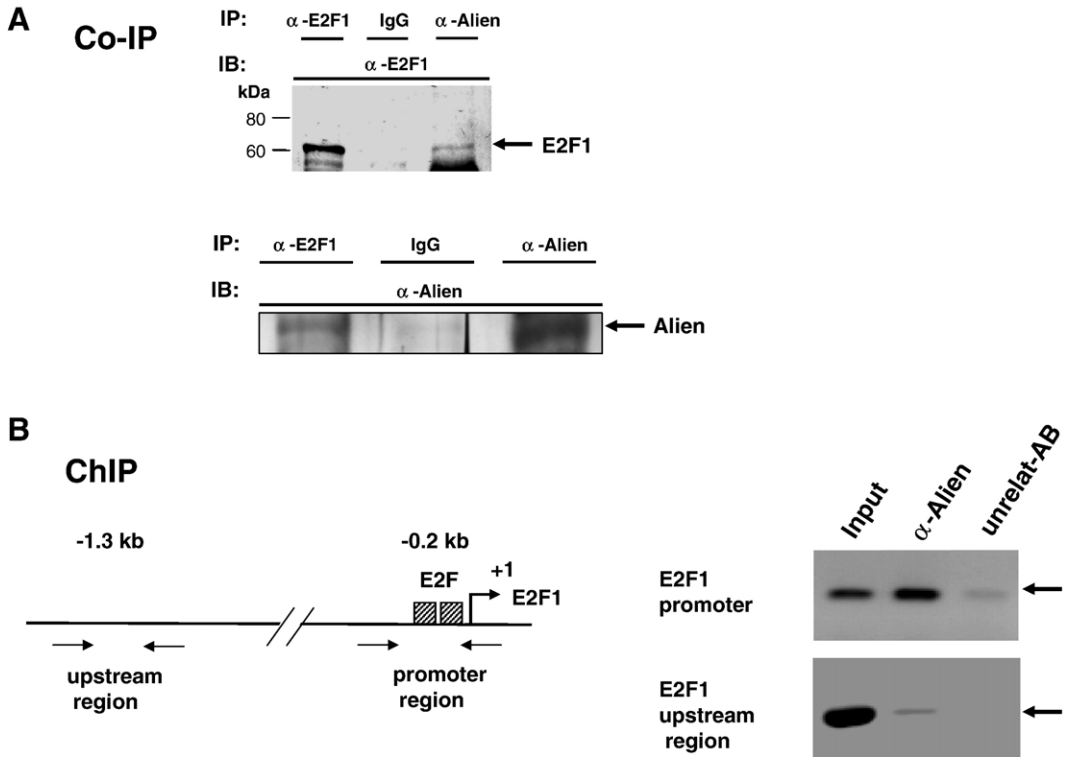

Fig. 1. Alien interacts with E2F1 and is recruited to the E2F1 promoter in vivo. (A) Co-IP experiments using for immunoprecipitation (IP) the anti-E2F1 or anti-Alien antibodies from U-2OS cell extracts and detection of the immunoprecipitate with anti-E2F1 antibody (upper panel) or the anti-Alien antibody (lower panel) in immunoblotting (IB). Arrows indicate the migration of the E2F1 and Alien proteins, respectively. IgG was used as unspecific and unrelated antibody. (B) ChIP experiments using LNCaP cells with anti-Alien or as control the anti-androgen receptor antibody as an unrelated antibody (unrelat. AB) and detection of the proximal E2F1 promoter containing the E2F binding sites (upper panel) or, as control, a 1.3-kb upstream region of the E2F1 gene (lower panel). Schematic view of E2F1 gene with the location of primers is indicated in the left panel. The arrows in the right panel indicate specific PCR products.

amounts (Fig. 2B). As control the expression vector for Gal alone was employed. As expected, transfection of Gal-E2F1 expression plasmid activates the reporter activity. However, expression of Alien leads to a dose-dependent inhibition of the reporter expression indicating that Alien inhibits the transactivation function of E2F1.

Since Alien is recruited to the E2F1 promoter and inhibits E2F1 transactivation function, we tested whether the endoge-

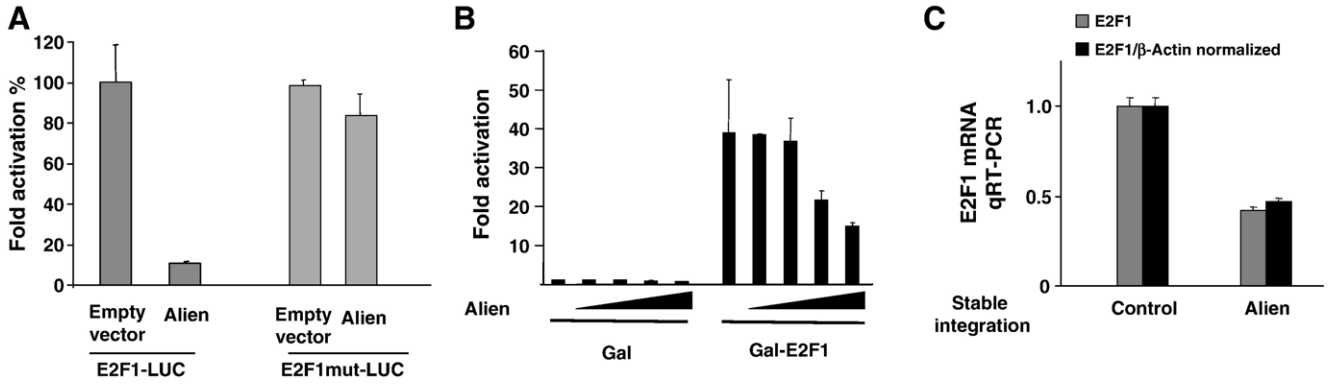

Fig. 2. Alien inhibits E2F1-mediated transactivation and E2F1 gene expression. (A) Transient expression of Alien (2 μg) in C33A cells lacking functional pRB inhibits specifically the 3xE2Fwt-luciferase reporter (1.5 μg) containing three E2F binding sites of the E2F1 promoter. As control the reporter with the three mutated E2F binding sites in the same promoter context (E2F1mut-luc) and the empty expression vector were used. The values obtained with the empty expression vectors were set arbitrarily as 100%. (B) The Gal-E2F1 fusion is repressed by Alien. The reporter pUAS4x-TATA-Luc (1.5 μg) was used to determine the effect of Alien on the Gal-E2F1 fusion in reporter assays. As control the expression vector for Gal alone was used, of which the value without Alien expression vector was set arbitrarily as one. Increasing amounts of Alien expression vector (0, 0.5, 1, 3 and 6 μg) were co-transfected with a constant amount of Gal-E2F1 (1μg) into C33A cells. The deviation from the mean of triplicate experiments is shown. (C) Quantitative real-time RT-PCR was used to detect the expression of the endogenous E2F1 gene expression. LNCaP cells stably transfected with Alien or as a control with the empty expression vector were employed. Both non-normalized (grey) and via beta-actin normalized mRNA values (black) are depicted. The amount of mRNA within the control cells was set as one. The deviation from the mean of two independent experiments is indicated.

nous E2F1 gene expression is repressed by Alien. For that purpose LNCaP cells were used for stable transfection with an Alien expression vector. After selection, stable cell clone pools were used and analyzed for expression of the endogenous E2F1 gene. Quantitative real-time RT-PCR was employed comparing the parental cells with stably transfected Alien expression vector (Fig. 2C). As internal control the beta-actin mRNA was used for normalization. Interestingly, cells stably transfected with Alien exhibited a reduced expression level of endogenous E2F1. This suggests that Alien inhibits the expression of E2F1.

Taking together, Alien inhibits transcription from the E2F1 promoter through its E2F binding sites, the transactivation of E2F1 and the endogenous E2F1 gene expression.

4.3. The expression level of Alien is modulated during cell cycle

To characterize further a possible association of Alien with the cell cycle, we analyzed whether the endogenous protein level of this corepressor varies during cell cycle progression. For that purpose, synchronized HeLa cells, as a model system for cell cycle phase analyses were employed. The cells were harvested in different cell cycle phases that were controlled by FACS analysis (Fig. 3C). Subsequently, the expression of Alien was detected by analyzing cell extracts with immunoblotting (Fig. 3A) and was normalized versus endogenous tubulin (Fig. 3B). Interestingly, the protein levels of Alien are higher in the G1, G1/S and G2 phases and lower in M- and S-phase. As control, the different cell cycle phases were determined by FACS analysis (Fig. 3C and supplemental data).

Thus, Alien protein levels change during the cell cycle phases and are highest in the G1, G1/S ands G2 phases.

4.4. Stable transfection of Alien reduces cell proliferation

Next, we considered whether the interaction of Alien with E2F1 and inhibition of E2F1 gene expression might influence cell proliferation. First, we have used LNCaP cells with stable transfected Alien (LNCaP-Alien). Comparing the growth properties of LNCaP-Alien cells with the parental cells, the cell pool with stably transfected Alien exhibited about a 2.3 fold reduced population doubling (Fig. 4A). Besides lower cell

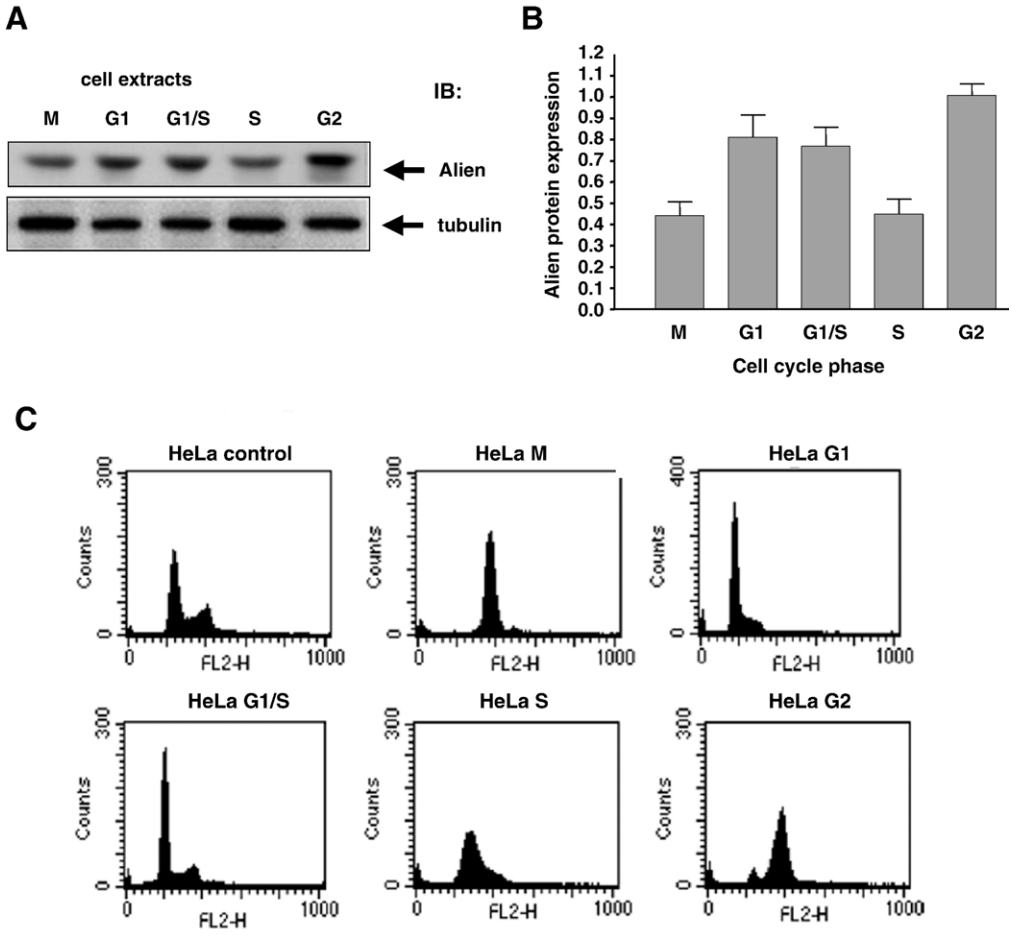

Fig. 3. Protein expression of Alien is regulated during cell cycle. (A) Synchronized HeLa cells were harvested in different cell cycle states and cell extracts were used for IB using anti Alien peptide antibody and anti-tubulin antiserum as loading control. (B) Western blots were quantified densitometrically and signal levels were normalized to the loading control and the Alien-levels at the G2-phase were set arbitrarily as 1. The deviation of the mean of two quantifications is indicated. (C) The distribution of synchronized HeLa cells at different cell cycle stages is indicated by FAC Scan analysis. Abbreviations: M=mitosis; G1=gap1-phase; G1/S=border gap1 to S-phase; S=S-phase; G2=gap2-phase.

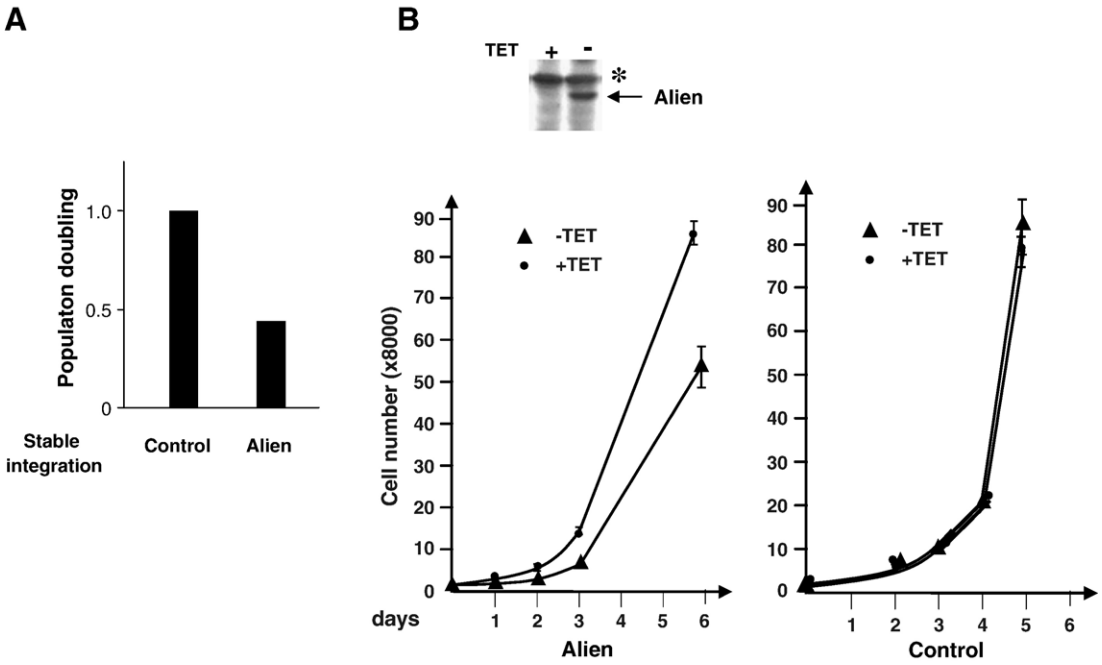

Fig. 4. Stable integration of Alien reduces cell proliferation. (A) LNCaP cells and LNCaP-Alien cells obtained after stable transfection and selection were compared for the cell proliferation and population doubling. (B) Tetracycline expression of Alien was achieved using the previously established NIH3T3 cells expressing the tet-activator. This tet-off system leads to expression of the target gene in the absence of tetracycline [30]. Stable integration of a tetracycline-regulated promoter linked to AU5-tagged Alien cDNA was introduced into these cells. The expression of Alien is shown by Western using an anti-AU5-tag antibody (top panel). Lower left panel: cell number was counted in either the presence or absence of tetracycline. The deviation of the mean of cell number is shown. Lower right panel: cell number of control cells stably integrated with the control plasmid as negative control [31], in either the presence or absence of tetracycline. *: unspecific band.

count, no apoptosis or detachment of cells was observed. In addition, we employed the established tetracycline regulatable system introduced in the immortalized NIH3T3 cells [30] and stably transfected Alien. Tetracycline (Tet) removal leads to the expression of the transfected gene (Tet-off system [38]). Single cell clones were selected and analyzed for their proliferation capacity in the absence or presence of tetracycline as assessed by cell count. In line with the previous data, stably Alien-transfected cells proliferated significantly less in the absence of tetracycline compared to cells grown in the presence of tetracycline (Fig. 4B). As control, cells were stably transfected with the control expression plasmid [31] (Fig. 4B). The population doubling was reduced by a factor of 1.5 fold in a tetracycline-dependent manner. Control cells with stable transfection of an unrelated gene did not exhibit a change in proliferation (data not shown). This suggests that the expression of Alien inhibits cell proliferation also in NIH3T3 cells.

Thus taken together, the data suggest that Alien inhibits E2F1 gene expression and cell proliferation.

5. Discussion

Based on our previous observation using a proteomic approach in which the corepressor Alien was identified as a novel interacting partner of the E2F1 transcription factor we intended to investigate the possible functional implication of this interaction.

Corepressors are defined as transcription factors that mediate or enhance the gene silencing of DNA bound transcription factors [39]. Characteristics of corepressors are that they act in the cell nucleus at chromatin level, and repress transcription by interacting with a transcription factor. Also, corepressors possess a silencing domain that mediates gene silencing. Corepressors could also play an important role in repressing the transactivation function of transcription factors. Alien has been shown to be corepressor for several nuclear receptors such as TR, VDR and DAX1 mediating gene repression [26–28].

Investigating promoter occupancy *in vivo* by ChIP assays revealed that Alien is recruited to the E2F1 promoter in the genomic region that includes the E2F binding sites. Since Alien repressed both the reporter encompassing E2F1 promoter sequences and the endogenous E2F1 gene expression, it suggests that repression by Alien is mediated through E2F binding sites. E2F binding sites are characterized by promiscuous recruitment of many E2F family members of transcription factors, but actually during late G1/S and S phase the E2F1 promoter is occupied and activated mainly by E2F1 and E2F3 *in vivo* [40].

In the case of E2F1 we here demonstrate that Alien is able to repress transcriptional activation mediated by the Gal-E2F1 fusion protein. Thus, this indicates that Alien is able to repress E2F1 activation function when tethered to the E2F1 promoter sequences. Taken together these data strongly suggest that Alien is a corepressor for E2F1.

The detailed molecular mechanism by which Alien inhibits E2F1-mediated transcriptional activity remains unclear. Interestingly, the transcriptional activity of E2F1 is also dependent on acetylation of E2F1 [15]. It may be based on blocking the

transactivation by hindering coactivator binding, or by the Alien-associated HDAC activity [26,29] on either chromatin level, or by de-acetylating E2F1 itself or a combination of these mechanisms.

It is well established that DNA-bound E2F1 remains repressed during the cell cycle beyond S-phase by direct binding of hypophosphorylated pRb recruiting chromatin-remodeling enzymes like among others SIN3 containing histone deacetylating complexes [41]. Interestingly, we recently also identified pRb and its relative p107 as interacting partners for the Alien corepressor [19]. A pRb pocket domain mutant (pRb_{706}) that lacks silencing function, thereby, also lacks Alien interaction. Besides pRb and p107, there are more examples of functionally interacting proteins that are common for Alien and E2F1. For example Sin3A and HDAC are associated with Alien and also were found to complex with E2F1 [26,29,42]. Thus, one could imagine that Alien may target a histone-modifying complex to promoter-tethered E2F1 transcription factor.

In addition, in the approach demonstrating repression of E2F promoter activity we employed human cervix carcinoma cells (C33A) that are known to lack functional pRb [43]. As a consequence, Alien seems to be able to repress E2F1 promoter independently from pRb. Such mechanisms have been described for E2F in association with other proteins such as for example prohibitin, HP-1γ, Max and others [41] and refs therein. This is of special interest explaining in part how some E2F-responsive genes remain repressed during S-phase (reviewed in [44]). Taken together, Alien may be an ambivalent protein able to repress E2F1-mediated transactivation in an Rb-independent fashion, but the existence of a ternary E2F1–Alien–pRb complex arbitrating E2F1 repression in special cellular constellations cannot be ruled out.

Furthermore, due to the positive feed back regulation of E2F1 on expression of its own gene, one can speculate that the level of Alien-mediated repression of E2F1 may also be dictated by Alien protein levels at different cell cycle phases. Therefore, on one hand it is tentative to speculate that higher Alien protein levels in G1, at the G1/S border and in G2 phase might inhibit more efficiently E2F-responsive gene transcription. On the other hand, lower Alien expression level in S-phase may contribute to de-repression of E2F-target genes. Interestingly, the protein expression profile of E2F1 seems to be inverted to that of Alien with highest levels of E2F1 in S-phase [24,25]. However, since the E2F1 promoter is regulated by multiple transcription factors, it is likely that a combination of these factors regulate the E2F1 gene expression. The role of lower Alien expression level observed in mitotic cells still remains unclear and may be unrelated to the pRb/E2F pathway.

Our data shown here indicate that Alien interacts with E2F1, is recruited to the E2F1 promoter sequences *in vivo* and represses E2F1-mediated transactivation as well as endogenous E2F1 gene expression. In line with this, stably expressed Alien reduces cell proliferation. Taken together this suggests that Alien has the characteristics of a bona fide corepressor for E2F1.

Acknowledgements

We are grateful to Dr. Bentley for providing Gal-E2F1 and Dr. P. Santisteban for the E2Fwt-Luc and E2Fmut-Luc reporter constructs, Dr. H. Blau for the pHRS-vectors and D.G. Schatz for the NIH3T3 cells. This work was supported by the grant of the German Research Foundation DFG BA 1457/2, the Academy of Finland and the Association of International Cancer Research (AICR).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2007.04.017.

References

[1] P.D. Adams, W.G. Kaelin Jr., The cellular effects of E2F overexpression, Curr. Top. Microbiol. Immunol. 208 (1996) 79–93.
[2] C. Stevens, N.B. La Thangue, The emerging role of E2F-1 in the DNA damage response and checkpoint control, DNA Repair (Amst) 3 (2004) 1071–1079.
[3] S. Inoshita, Y. Terada, O. Nakashima, M. Kuwahara, S. Sasaki, F. Marumo, Regulation of the G1/S transition phase in mesangial cells by E2F1, Kidney Int. 56 (1999) 1238–1241.
[4] L.A. Bell, K.M. Ryan, Life and death decisions by E2F-1, Cell Death Differ. 11 (2004) 137–142.
[5] M.E. Crosby, A. Almasan, Opposing roles of E2Fs in cell proliferation and death, Cancer Biol. Ther. 3 (2004) 1208–1211.
[6] D. Ginsberg, E2F1 pathways to apoptosis, FEBS Lett. 529 (2002) 122–125.
[7] N.B. La Thangue, The yin and yang of E2F-1: balancing life and death, Nat. Cell Biol. 5 (2003) 587–589.
[8] A.C. Phillips, K.H. Vousden, E2F-1 induced apoptosis, Apoptosis 6 (2001) 173–182.
[9] I. Palmero, G. Peters, Perturbation of cell cycle regulators in human cancer, Cancer Surv. 27 (1996) 351–367.
[10] K. Helin, Regulation of cell proliferation by the E2F transcription factors, Curr. Opin. Genet. Dev. 8 (1998) 28–35.
[11] J. DeGregori, D.G. Johnson, Distinct and overlapping roles for E2F family members in transcription, proliferation and apoptosis, Curr. Mol. Med. 6 (2006) 739–748.
[12] E. Neuman, E.K. Flemington, W.R. Sellers, W.G. Kaelin Jr., Transcription of the E2F-1 gene is rendered cell cycle dependent by E2F DNA-binding sites within its promoter, Mol. Cell. Biol. 14 (1994) 6607–6615.
[13] D. Trouche, A. Cook, T. Kouzarides, The CBP co-activator stimulates E2F1/DP1 activity, Nucleic Acids Res. 24 (1996) 4139–4145.
[14] P.S. Goldman, V.K. Tran, R.H. Goodman, The multifunctional role of the co-activator CBP in transcriptional regulation, Recent Prog. Horm. Res. 52 (1997) 103–119 (discussion 119–20).
[15] M.A. Martinez-Balbas, U.M. Bauer, S.J. Nielsen, A. Brehm, T. Kouzarides, Regulation of E2F1 activity by acetylation, EMBO J. 19 (2000) 662–671.
[16] W.G. Kaelin Jr., W. Krek, W.R. Sellers, J.A. DeCaprio, F. Ajchenbaum, C.S. Fuchs, T. Chittenden, Y. Li, P.J. Farnham, M.A. Blanar, et al., Expression cloning of a cDNA encoding a retinoblastoma-binding protein with E2F-like properties, Cell 70 (1992) 351–364.
[17] K. Helin, E. Harlow, A. Fattaey, Inhibition of E2F-1 transactivation by direct binding of the retinoblastoma protein, Mol. Cell. Biol. 13 (1993) 6501–6508.
[18] O. Stevaux, N.J. Dyson, A revised picture of the E2F transcriptional network and RB function, Curr. Opin. Cell Biol. 14 (2002) 684–691.
[19] N. Escher, R. Kob, S.P. Tenbaum, M. Eisold, A. Baniahmad, F. von Eggeling, C. Melle, Various members of the E2F transcription factor family

interact in vivo with the corepressor alien, J. Proteome Res. 6 (2007) 1156–1164.
[20] R. Schneider, A.J. Bannister, T. Kouzarides, Unsafe SETs: histone lysine methyltransferases and cancer, Trends Biochem. Sci. 27 (2002) 396–402.
[21] R. Ferreira, I. Naguibneva, L.L. Pritchard, S. Ait-Si-Ali, A. Harel-Bellan, The Rb/chromatin connection and epigenetic control: opinion, Oncogene 20 (2001) 3128–3133.
[22] D.G. Johnson, J.K. Schwarz, W.D. Cress, J.R. Nevins, Expression of transcription factor E2F1 induces quiescent cells to enter S phase, Nature 365 (1993) 349–352.
[23] L. Wu, C. Timmers, B. Maiti, H.I. Saavedra, L. Sang, G.T. Chong, F. Nuckolls, P. Giangrande, F.A. Wright, S.J. Field, M.E. Greenberg, S. Orkin, J.R. Nevins, M.L. Robinson, G. Leone, The E2F1-3 transcription factors are essential for cellular proliferation, Nature 414 (2001) 457–462.
[24] J.E. Slansky, Y. Li, W.G. Kaelin, P.J. Farnham, A protein synthesis-dependent increase in E2F1 mRNA correlates with growth regulation of the dihydrofolate reductase promoter, Mol. Cell. Biol. 13 (1993) 1610–1618.
[25] C. Sardet, M. Vidal, D. Cobrinik, Y. Geng, C. Onufryk, A. Chen, R.A. Weinberg, E2F-4 and E2F-5, two members of the E2F family, are expressed in the early phases of the cell cycle, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 2403–2407.
[26] U. Dressel, D. Thormeyer, B. Altincicek, A. Paululat, M. Eggert, S. Schneider, S.P. Tenbaum, R. Renkawitz, A. Baniahmad, Alien, a highly conserved protein with characteristics of a corepressor for members of the nuclear hormone receptor superfamily, Mol. Cell. Biol. 19 (1999) 3383–3394.
[27] B. Altincicek, S.P. Tenbaum, U. Dressel, D. Thormeyer, R. Renkawitz, A. Baniahmad, Interaction of the corepressor Alien with DAX-1 is abrogated by mutations of DAX-1 involved in adrenal hypoplasia congenita, J. Biol. Chem. 275 (2000) 7662–7667.
[28] P. Polly, M. Herdick, U. Moehren, A. Baniahmad, T. Heinzel, C. Carlberg, VDR-Alien: a novel, DNA-selective vitamin D(3) receptor–corepressor partnership, FASEB J. 14 (2000) 1455–1463.
[29] U. Moehren, U. Dressel, C.A. Reeb, S. Vaisanen, T.W. Dunlop, C. Carlberg, A. Baniahmad, The highly conserved region of the co-repressor Sin3A functionally interacts with the co-repressor Alien, Nucleic Acids Res. 32 (2004) 2995–3004.
[30] P. Shockett, M. Difilippantonio, N. Hellman, D.G. Schatz, A modified tetracycline-regulated system provides autoregulatory, inducible gene expression in cultured cells and transgenic mice, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 6522–6526.
[31] F.M. Rossi, O.M. Guicherit, A. Spicher, A.M. Kringstein, K. Fatyol, B.T. Blakely, H.M. Blau, Tetracycline-regulatable factors with distinct dimerization domains allow reversible growth inhibition by p16, Nat. Genet. 20 (1998) 389–393.
[32] A.I. Protopopov, J. Li, G. Winberg, R.Z. Gizatullin, V.I. Kashuba, G. Klein, E.R. Zabarovsky, Human cell lines engineered for tetracycline-regulated expression of tumor suppressor candidate genes from a frequently affected chromosomal region, 3p21, J. Gene Med. 4 (2002) 397–406.
[33] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[34] W. Krek, J.A. DeCaprio, Cell synchronization, Methods Enzymol. 254 (1995) 114–124.
[35] R. Lehmann, C. Melle, N. Escher, F. von Eggeling, Detection and identification of protein interactions of S100 proteins by ProteinChip technology, J. Proteome Res. 4 (2005) 1717–1721.
[36] F. Goeman, D. Thormeyer, M. Abad, M. Serrano, O. Schmidt, I. Palmero, A. Baniahmad, Growth inhibition by the tumor suppressor p33ING1 in immortalized and primary cells: involvement of two silencing domains and effect of Ras, Mol. Cell. Biol. 25 (2005) 422–431.
[37] J. Blau, H. Xiao, S. McCracken, P. O'Hare, J. Greenblatt, D. Bentley, Three functional classes of transcriptional activation domain, Mol. Cell. Biol. 16 (1996) 2044–2055.
[38] M. Gossen, H. Bujard, Tight control of gene expression in mammalian cells by tetracycline-responsive promoters, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 5547–5551.
[39] L.J. Burke, A. Baniahmad, Nuclear hormone receptor co-repressors, FASEB J. 14 (2000) 1876–1888.
[40] K. Araki, Y. Nakajima, K. Eto, M.A. Ikeda, Distinct recruitment of E2F family members to specific E2F-binding sites mediates activation and repression of the E2F1 promoter, Oncogene 22 (2003) 7632–7641.
[41] M.V. Frolov, N.J. Dyson, Molecular mechanisms of E2F-dependent activation and pRB-mediated repression, J. Cell Sci. 117 (2004) 2173–2181.
[42] A. Lai, B.K. Kennedy, D.A. Barbie, N.R. Bertos, X.J. Yang, M.C. Theberge, S.C. Tsai, E. Seto, Y. Zhang, A. Kuzmichev, W.S. Lane, D. Reinberg, E. Harlow, P.E. Branton, RBP1 recruits the mSIN3–histone deacetylase complex to the pocket of retinoblastoma tumor suppressor family proteins found in limited discrete regions of the nucleus at growth arrest, Mol. Cell. Biol. 21 (2001) 2918–2932.
[43] M. Scheffner, K. Munger, J.C. Byrne, P.M. Howley, The state of the p53 and retinoblastoma genes in human cervical carcinoma cell lines, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 5523–5527.
[44] A.P. Bracken, M. Ciro, A. Cocito, K. Helin, E2F target genes: unraveling the biology, Trends Biochem. Sci. 29 (2004) 409–417.